Supporting Information

The Phase Diagram of Charged Colloidal *Lipid A-Diphosphate* Dispersions

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S-1. Preparation of *lipid A-diphosphate*. - All chemicals were of ACS analytical grade, and triple-distilled water was used throughout for the solutions and dilutions. The *lipid A-diphosphate* was prepared from lipopolysaccharide (LPS) from *E. coli* bacteria (strain 715, Re-mutant). LPS was isolated as the acetate-soluble fraction following the modified procedure of Hansen & Phillips [1] It was subsequently purified by HPLC (Beckman Gold) using a linear gradient for elution by 0.05 M sodium acetate in the presence of 0.001 M acetic acid, or citrate (1.0 mM) at 4°C, which contained 0.001 M acetic acid and 0.015 M ammonium acetate [2]. The flow rate was generally 1.5 mL/h. Peaks were monitored using a differential refractive index detector, coupled to the light scattering apparatus, which simultaneously calculated the weighted-average molecular weights of the desired peaks. From angle dependency (five different angles) of each peak, the weighted-average radius of gyration and its deviation were evaluated. Size-exclusion chromatography (SEC) was performed as described previously [3]. The average-weighted molecular weights were

determined for *lipid A-diphosphate,* employing the above preparation techniques and were as follows: $\overline{M_w}$ = (10.5 ± 0.9) x 10^6 g/mol (light scattering detector) and $\overline{M_w}$ = (11.0 ± 0.5) x 10^6 g/mol (SEC). The material was analyzed by MALDI-TOF-MS, negative ion electron-spray/MS and GC/MS[4]. The elemental analysis of the phosphate content, and amino sugars were performed as described in [5] and also according to Dische [6] using lyophilized *lipid A-diphosphate* material.

S-2. Preparation of *lipid A-diphosphate* dispersions.- Pure *lipid A-diphosphate* material was dialyzed against several changes of 0.5 mM NaCl pH 5.6 (4°C) and after which any particulate matter was removed by means of filtration through a Millipore filter (exclusion particle-size diameter 2.5 μm). The low-ionic-strength form of *lipid A-diphosphate* (300 μg/mL) was diluted to a concentration of 100 μg/mL and then washed using a tangential ultrafiltration module (Schleicher and Schüll, Germany). The module removed the water from the sol and replaced it with de-ionized water, continuously reducing the ionic strength of the sol. Using this procedure, a batch of *lipid A-diphosphate* was washed until the water leaving the system had a conductivity close to that of the de-ionized water used in the tangential ultrafiltration module. At this stage, the batch of *lipid A-diphosphate* was divided into two portions. One portion was concentrated to a volume fraction of $\phi = 1.2 \times 10^{-2}$, while the other portion was diluted to a volume fraction of $\phi = 1.2 \times 10^{-3}$, with both portions having an ionic strength of 2.5×10^{-5} M and a pH of 5.6. This technique permits the examination of two lipid A-diphosphate systems that differ only in volume fraction. The ion-exchange resins from BioRad were treated with the same Millipore water as before the exchange experiments were performed, e.g. OH^- to H^+. This means, a fixed quantity of water was used and the effluent was re-circulated, which ensured that the equilibrium system conditions, i.e. the ionic strength and the pH, were always maintained. All of the solutions were transparent. A minimum ionic strength of ~10^{-4} M was reached after 12 hrs, at which stage all small ions except the H^+ or

OH^- were removed. The concentration of the *lipid A-diphosphate* was determined by weighing a sample before and after drying in an oven at 100° C under a stream of N_2 (99.99%) for 2 hrs. Conversion of units from mass fraction to concentration in μg of *lipid A-diphosphate*/liter, c, and volume fraction, ϕ ($\phi = \nu \cdot c$, where ν is the partial specific volume in mL/g) was made by assuming a density of the dry *lipid A-diphosphate* of 1.39 ± 0.075 g/cm^3.

S-3: Light Scattering. - Static-light-scattering (LS) experiments were performed using an instrument described previously [3]. The light source was a polarized laser beam (35 mW He-Ne) of a wavelength λ_0 = 637.8 nm. The incident beam was vertically polarized and the intensity adjusted by a combination of neutral density filters, including a half-wave plate and a polarizer, in order to achieve maximum amplitude within the linear response range of the photomultiplier. Static-light-scattering (LS) measurements of dilute suspensions of *lipid A-diphosphate* were performed at Q values between 8.0×10^{-3} and 3.5×10^{-2} nm^{-1}. The alignment of the apparatus, particularly for the angular dependence of the scattered intensity, was tested using an aqueous suspension of well-characterized colloidal polystyrene particles (Sigma-Aldrich), and the form factor, *P(Q)*, was verified with Mie scattering theory. Average intensities were obtained from 20 individual runs at each scattering angle, and I(Q) was corrected for variations in the scattering volume.

Scattering intensities were measured for P(Q) in the above-mentioned Q range in 0.1° steps for angles around the first intensity maximum, 0.15° steps at the second intensity maximum, and 2.5° for higher angles. The accessible range was limited to the volume fraction range of *lipid A-diphosphate* ($2.0 \times 10^{-4} \leq \phi \leq 3.5 \times 10^{-4}$) in order to avoid Bragg reflections, which would superimpose on the scattering from the coexisting liquid phase. However, the samples of *lipid A-diphosphate* form colloidal crystals at higher concentrations, $\phi_c > 3.5 \times 10^{-4}$ and above $\phi > 3.5 \times 10^{-3}$ to crystal sizes up to 200 μm. For *lipid A-diphosphate* dispersions the absorbance at 556 nm due to scattering was 0. 15.

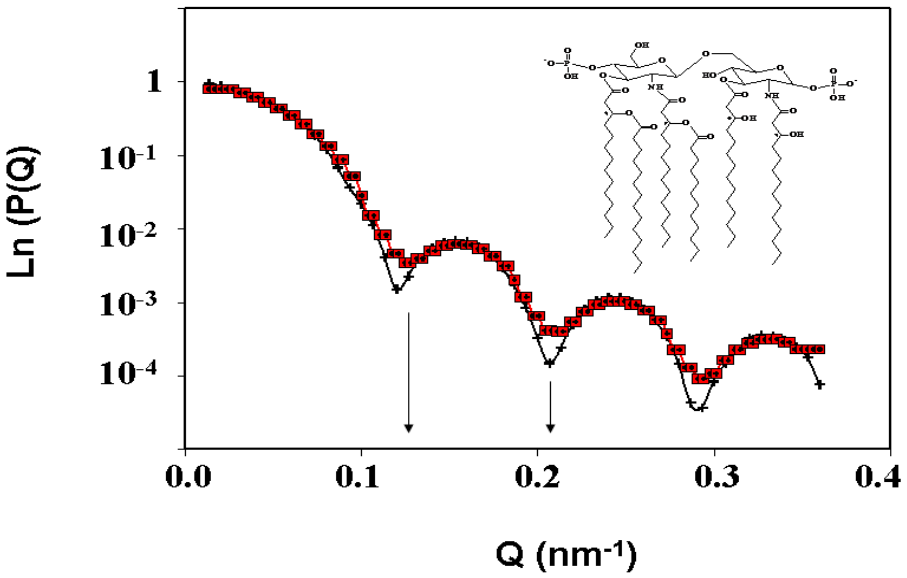

Figure 1 of S-3: Typical static-light-scattering curve for the liquid phase of a *lipid A-diphosphate* aqueous dispersion at a volume fraction ϕ = 2.0 x 10^{-4}, and I = 0.5 mM, (*red*). Plotted as the form factor *(P)* vs. the scattering vector *Q*; drawn curve (*black)* is the Rayleigh-Gans-Debye (RGD) form factor with polydispersity σ = 5.2 %, R = 35 nm, and m = 1.057. The arrows show the positions of the first and second minima in the RGD approximation. **Inset**: Chemical structure of *lipid A-diphosphate*.

S-4: Small-Angle X-Ray scattering.- Small-angle X-ray scattering (SAXS) experiments were performed on a small-angle X-ray diffraction & scattering setup with a linear position-sensitive detector as described previously, which includes data handling and processing procedures. [3,7] Data were recorded for Q-values as low as 0.02 nm^{-1} (Cu Kα radiation (λ=0.154 nm). The beam was Kα/Kβ filtered and focused in horizontal and vertical direction by total reflection from curved Franks mirrors. The height of the X-ray beam on the sample was set at 1.0 mm, and a system of moving horizontal slits in front of the detector reduced the effects of beam height; the slits were adjusted to form a horizontal entrance slit of approximately 3.5 mm. The liquid samples were placed in 1.5 mm thick cell holders surrounded by a Kepton film. For the crystalline phases, the samples were continuously

rotated over an area of 35 nm^2 in order to obtain a good powder average. The scattering vector Q varied from 0.03 nm^{-1} to 0.4 nm^{-1}, and the dQ/Q resolution was of the order of 0.025. The samples were thermostated using a water bath (Heraeus, Germany) to 20 ± 0.1°C°.

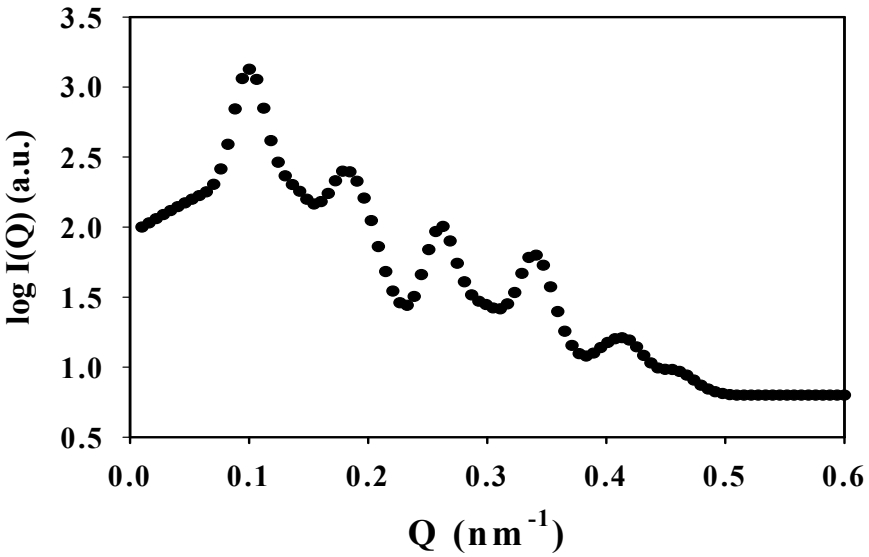

Figure 1 of S-4: Typical SAXS-profile for *lipid A-diphosphate* dispersion containing BCC-(*Im3m)* type crystals with a = 37.6 nm, a volume fraction of $\phi = 3.5 \times 10^{-4}$ and ionic strength I = 0.5 mM NaCl (20°C).

The peaks observed, for *lipid A-diphosphate* dispersion ($\phi = 3.5 \times 10^{-4}$), which were assigned to a BCC lattice ($h^2 + k^2 + l^2$: 2, 4, 6, 8 & 10, and $h + k + l$ = 2n) with a = 37.6 nm and possible space group of *Im3m* [8]. The molecular weight calculations for this phase of *lipid A-diphosphate* were made by using the (110) diffraction planes, $d = d_{110} = a/(2)^{1/2}$. For this BCC lattice type, the estimated molecular weight was found to be approximately 10.1×10^6 g/mol.

The cubic phases were all non-lamellar under these experimental conditions and none belonged to *Pn3m* space group, with a spacing ratio of 1, 1:√2, 1:3; 1:√4, 1:6, etc. , or to a *Pm3n* structure with diffraction lines of the following ratios: √2, √4, √5, √6, √8 and √10, etc. Because of an insufficient number of reflections, making a conclusive analysis so that a

specific space group could be determined was not possible. Both *Im3m* and *Pm3n* structures were possibilities and the structure consists of disjoined clusters of *lipid A-diphosphate* separated by a continuous film of water. The body-centered cubic phase is generally present at higher water contents and low ionic strengths [3], where the structure may be modeled by a packing of clusters of identical rigid spheres embedded in water. The closest packing of these spheres takes place in the [1,1,1] array and the radius of the closely packed rigid sphere is $R_{sp} = (\sqrt{3}/4 \cdot a) = 16.4$ nm. In contrast to the *Im3m,* phase the *Pm3n* is considered to contain two types of clusters or micelles [9].

A space-filling polyhedra cell model contains the aliphatic core and is surrounded by the hydrophilic disaccharide region with its accompanying phosphates, [9] may be represented by the space group *Pm3n.* This would give rise to a hexagonal or rectangular direct phase, where the aliphatic cores would no longer be spherical, oblate or cylindrical. The resulting structure would be one of chains, which stack and fit the curvature to the interfaces, maintaining a constant thickness of the hydrophobic part between neighboring clusters. The phosphates at the end and at the outside of the disaccharide rings can form strong hydrogen bonds to adjacent (opposite) interfaces. This inter- and intra- hydrogen-bonding scheme with water could stabilize the phase. This would also be a suitable description for *lipid A-monophosphate* [10], the Ca^{2+} and K^{+}-salts of *lipid A-diphosphate* [11] and the rod-like ordered assembly of *lipid A-diphosphate* at pH 8.5 at low ionic strength [12]. It would also be applicable to dispersions of *lipid A-diphosphate* containing methanol (or n-butanol)/toluene/water/1 mM NaCl at 25°C, [13] which form an extensive hydrogen-bonded network at low water and salt content.

From a model point of view for the assembly of *lipid A-diphosphate* in aqueous dispersions, the polyhedral assemblies for both *Im3m* and *Fm3m* (glassy phase) may be reconciled as a packed and truncated octahedral and rhombic dodecahedra. Luzzati et al. has

described their relationship, octahedral vs. rhombic dodecahedra to non-congruent infinite periodic minimal surfaces [14].

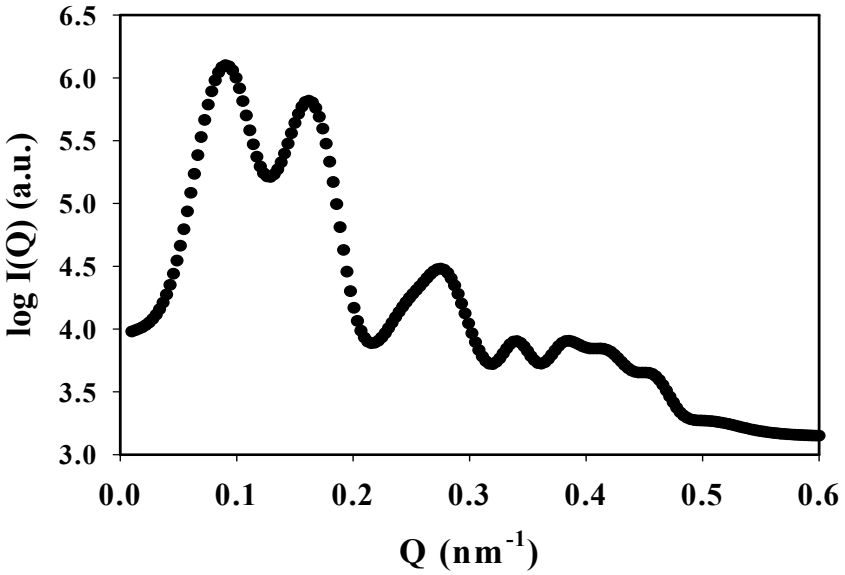

Figure 2 of S-4: Typical SAXS profile for a *lipid A-diphosphate* dispersion obtained for the colloidal FCC (*Fd3m)* type crystals with a = 57.5 nm at $\phi = 5.0 \times 10^{-4}$, and I = 0.5 mM NaCl (20°C).

From the SAXS experiments at a volume fraction ϕ = 5.0 x 10-4 and an ionic strength I = 0.5 mM NaCl) sharp peaks were recorded, indicating the presence of long-range order. A tentative assignment of the observed peaks of *lipid A- diphosphate,* Q_{hkl}, to the crystal planes was made by comparing the observed peak vectors with the calculated ones according to equation S-4-1:

(S-4-1) $$Q = 2\pi/a\ (h^2 + k^2 + l^2)^{1/2}.$$

In the above equation h, k and l are the Miller's indices and a is the unit cell size. Most, but not all, of the peaks may be assigned to the reflections of an FCC lattice ($h^2 + k^2 + l^2$: 3, 8, 11, 12 & 16) as indicated in Figure 1 d, and Figure 2 of S-4 with a = 57.5 nm, and appear to correspond to the space group *Fd3m* [8]. The reflections systematically absent are the $h + k$, k +l

and $h + l \neq 2n$ for the general reflections (*hkl*) and $k + l \neq 4n$ for the *(0kl)* zone. The absences reinforce the argument that the lattice type is face-centered and two space groups are possible, namely *Fd3* and *Fd3m* [8]. The two space groups are also centrosymmetric and belong to the Laue classes *m3* and to *m3m*. Note: *Fd3m* corresponds to special positions of *Fd3*.

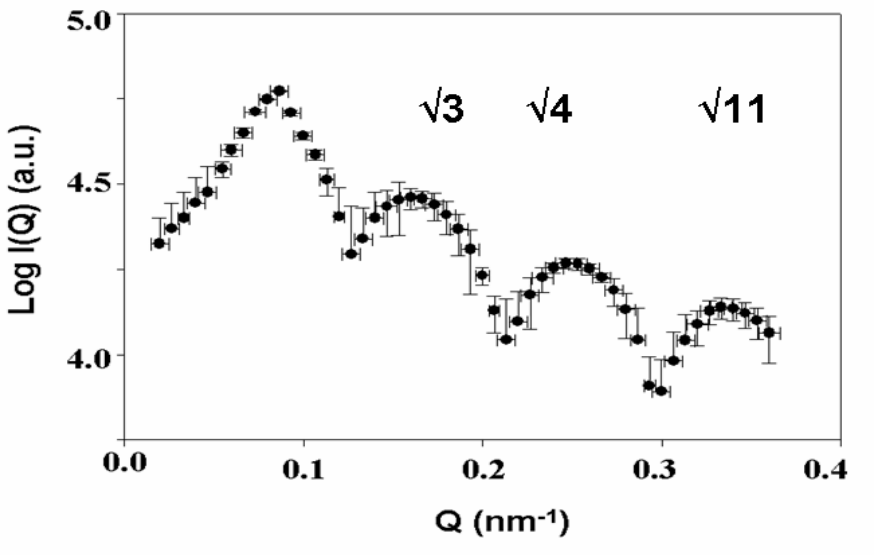

Figure 3 of S-4: SAXS profile for the glass phase of *lipid A-diphosphate* dispersions for a volume fraction of $\phi = 4.0 \times 10^{-4}$, and ionic strength of I = 10 μM HCl (20°C).

The spectra recorded for the glassy phase show reasonable correlation with the *Fm3m* space group, which could be a structure based on the F-RD minimal surface. However, the quality of the spectra is not sufficient to make conclusive deductions as to space group at present.

S-5: Electron Microscopy: Particle size distributions were characterized by imaging the particles at a voltage of 300 kV using a transmission microscope (JEOL 3010).

S-6: ***References:***

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